AD 678896

TRANSLATION NO. 1032

DATE: July 1968

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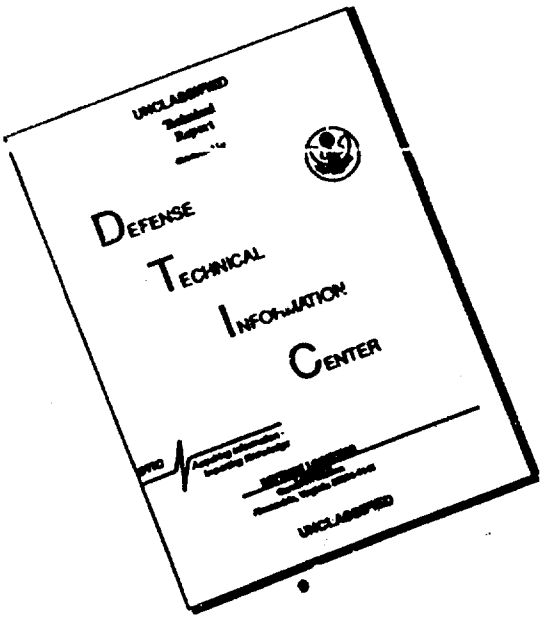

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A RAPID METHOD OF OBTAINING ANTIBODIES LABELLED WITH FLUORESCENT STAINS

[Following is the translation of an article by P.K. Tabakov, Ye.V. Chibrikova, I.I. Shurkina, and Ye.I. Vel'ner (All-Union Scientific Research Institute "Mikrob" (Saratov)) in the Russian-language publication Zhurnal Mikrobiologii, Epidemiologii, i Immunologii (Journal of Microbiology, Epidemiology, and Immunology), Vol XXXIII, No 10, Moscow, 1962. Additional bibliographic information accompanies each article.]

Over the last four years (1957-1960), the Soviet press has carried a number of reports indicating the possibility of rapid bacterioscopic identification of grampositive and gramnegative pathogenic bacteria in various objects with the aid of fluorescent antibodies.

These reports described the Coons and Kaplan (1950) methods of obtaining and employing fluorescent globulins with the application of domestic luminescent dyes and apparatus. For the purpose of isolating antibodies (globulins) from immune serums, the authors employed the method of repeated salting out with neutral salts -- ammonium sulfate or sodium sulfate with subsequent dialysis against buffered solutions of 0.15 M NaCl. The same procedure was also used for the purification of fluorochrome-labelled antibodies.

The time expended on the preparation of the labelled antibody reached 3-4 weeks, most of this spent on dialysis. In order to shorten the time for the preparation of conjugates, various modifications of the method of their purification were devised. Dineen and Ada (1957) suggested the removal of the fluorochrome excess by ethylacetate extraction; Dashkevich et al. (1959) for the same purpose used acetone extraction at low temperatures; processing activated charcoal and liver powder has also been suggested.

In the present study we attempted to curtail as much as possible the time for preparation of labelled antibodies through

the exclusion of the salting-out stage followed by dialysis as the longest operation. At the same time, we attempted to develop a technique which could be used under laboratory conditions without special equipment.

We carried out several experiments for the comparative study of the quality of fluorochromically-labelled antibodies prepared in various ways from agglutinating anti-cholera and anti-plague serums (the serums were obtained from horses and rabbits immunized by the corresponding microbes).

The isolation of antibodies for each serum was carried out simultaneously by three methods: 1) 3-fold salting out of globulins with ammonium sulfate followed by dilution and dialysis against a buffered 0.15 M NaCl solution (Glubokina, Kabanova, Levina, and Pishchurina, 1960); 2) salting out with sodium sulfate (Dashkevich, D'yakov, Yermakov, Ivanova, Mayboroda, 1959); 3) precipitation of globulins with alcohol according to the Cohn principle (1941, 1944, 1949) and the technology developed at the Moscow Institute imeni Mechnikov (Nechayeva and Ponomareva, 1956).

The isolated globulins were labelled according to the Coons and Kaplan technique (1950) with fluorescein isocyanate. Conjugate (labelled antibody) purification was carried out by various methods: 1) 3- or 4-fold reprecipitation with ammonium sulfate followed by dialysis, as described in the article by Glubokina et al.; 2) reprecipitation with sodium sulfate (Dashkevich et al.); 3) low-temperature alcohol precipitation (Cohn) followed by solution in buffer solvent 0.15 M NaCl (pH = 9.0).

The relative advantages of each of the resulting conjugates were determined by their serological activity as manifested in the agglutination reaction, and mainly in their ability to produce the specific luminescence of homologous bacteria which was detected in the luminescent microscopy of smears treated with the corresponding conjugates (description of method will be found in the article by Chibrikova et al.).

The results of the completed experiments also made it possible to arrive at the conclusion that the antibodies isolated from the same serum by these methods and labelled with fluorescein isocyanate of a single series, did not differ essentially with respect to serological activity.

At the same time, it is necessary to defer to alcohol precipitation by the Cohn method, since the isolation of the common globulin and γ - globulin from the serum in this case required 1-2 days against 5-7 days with salt precipitation followed by dialysis. The same time was required for the purification of labelled antibodies through alcohol precipitation. The total amount of time saved by employing alcohol precipitation in place of salting out was 10-14 days. Unfortunately, the method of alcohol precipitation is unavailable to many laboratories, since the precipitation of globulins

with alcohol by Cohn's method requires a refrigeration room with a working temperature of -5° and a special device for cooling the alcohol down to -22°.

According to some published data, the euglobulin fractions of immune serums possess the properties of antibodies. Thus, with low-temperature alcohol precipitation Markovich (1939) isolated an euglobulin fraction having antibody properties from anti-plague serums. Gaurovitts (1953) in his book The Chemistry and Biology of Proteins notes that the euglobulin fraction of immune serums contains a considerable portion of antibodies. Krauya (1959), in describing serums against swine erysipelas, proves that γ-globulins should not be regarded as carriers of antibodies in concentrated serums; the best concentrated serums with the highest content of biologically active proteins were obtained by him in the salting out of euglobulin.

On the basis of these premises, we undertook an attempt at testing euglobulin fractions for the preparation of fluorochromically-labelled antibodies.

The isolation of the euglobulin fraction from serums is possible not only by the methods of alcohol or saline precipitation, but by a simpler method as well. With a high dilution of the serum with distilled water, a considerable portion of the globulins is precipitated out. This is the portion which was called euglobulin (Hofmeister and Pick, 1902). Lenderz (1925) precipitated euglobulins through the 10-fold dilution of a serum with a weak acetic acid solution. The proteins thus precipitated were called labile globulins by the author. Simon (1954), Fife and Muschel (1959), and Kotlyarov (1960) precipitated euglobulins through multiple (10- to 21-fold) dilution with distilled water and acidification of the dissolved serum with hydrochloric acid to pH = 6.4-6.5.

In connection with the above, the water method of precipitation and purification of antibodies (euglobulins) seemed most promising to us, and we realized it in the following way.

Anti-cholera or anti-plague serum cooled to 1-2° was diluted in a 1:14 ratio with distilled water cooled to 1-2°. Up to 1 ml of the resulting solution was placed in a series of test tubes to which were added increasing amounts (0.05, 0.10, 0.15 ml, etc.) of M/50 acetate buffer with pH = 4.65. The test tube with the maximum clouding (isopoint) was noted.

After this, a quantity M of acetate buffer of pH = 4.65 was added to the basic mass of the serum solution, which was mixed an allowed to remain for 30 minutes in ice water to permit the formation of a precipitate, after which the euglobulin was separated from the solution by 5-10 minutes of centrifuging at 5000 rpm in a centrifuge placed in a refrigerator with a temperature of 4-5°.

The centrifuging was carried out in weighed test tubes (cartridge type). The supernatant fluid was decanted and the globulin collected at the bottom was weighed and dissolved in 1.5 M NaCl in an amount equal to 0.1 (by weight in grams) of the precipitate. The loose euglobulin precipitate retained considerable quantities of the matrix solution (up to a 20-fold amount by weight), so that with the addition of small amounts of 1.5 M NaCl and mixing it easily went into solution. The protein content in such solutions, determined with the aid of a refractometer, varied between 6.4 and 9.3%.

The isolated euglobulin fractions of the immune serums were studied with the aid of the EFA-1 paper electrophoresis apparatus. The electrophoresis was carried out on strips of "Bystraya" brand chromatographic paper in a veronal-medinal buffer with pH = 8.6, an ion force of 0.1, a current of 0.5 mA and a voltage within 7-8 volts/cm of strip length.

The analysis showed that the globulins in their electrophoretic composition were γ-globulins.

The labelling of the isolated antibodies was carried out by means of fluorescein isocyanate preparations of the No 35 series and the I and II isomers of fluorescein isothiocyanate obtained from the Chemical Reagents Institute. The fluorescein isocyanate labelling was carried out according to the Coons and Kaplan procedure. As regards the labelling of antibodies with isothiocyanate, we used the method of Riggs (1959) and the simpler technique of Marshall (1958). The latter turned out to be the more convenient and effective one.

Since it has not been described in the domestic literature, we shall outline it briefly here.

The euglobulin fraction of the immune serum was dissolved in 0.15 M NaCl and a carbonate-bicarbonate buffer 0.5 M (pH = 9.0) in such a way that the final solution contained 10 mg/ml protein and 10% buffer solution. After cooling of the solution to 4°, 0.05 mg of dry fluorescein isothiocyanate were added per mg of protein. The mixture was placed in a flask or wide test tube with a magnetic mixer and immersed in an ice bath. The mixing was continued for 18 hours, after which the conjugate was subjected to purification from an excess of dye.

The purification of the labelled antibody (conjugate) was carried out in the same way as the isolation of euglobulin from the immune serum. Usually, it was sufficient to carry out a single water precipitation. But at times, in order to remove the fluorochrome excess, 2-fold precipitation was needed. The completeness of precipitation was established from the residual luminescence of the centrifuged material upon illumination with ultraviolet rays. The precipitate of the fluorochromically labelled euglobulin was dissolved in 0.2 M Na_2CO_3 taken in an

amount (in milliliters) equal to the number of grams of precipitate. The protein content in the resulting solution was measured by the microkjeldal method followed by isothermic distillation in plastic Conway cups (Tabakov).

In toto, all of the operations involved in the isolation of the globulin from the serum, its fluorochromic labelling, and removal of excess dye from the labelled globulin required 3-4 days instead of the 21-28 days necessary with the Coons-Kaplan method.

To compare the properties of euglobulins obtained by water precipitation, we prepared an purified common-globulin fractions by Cohn water-alcohol precipitation. Both globulins were labelled with the same fluorochrome. After determination of the protein content, the labelled antibodies were used for dyeing smears from homologous and other species of bacteria (for checking specificity).

The results of dyeing plague and cholera agents with labelled antibodies are given in the table, whence we see that the antibodies labelled with fluorescein isocyanate, judging from the protein content in the working solution (the "working solution" being the maximum solution with which the coloring of homologous bacteria does not differ in intensity from the coloring obtained by treating the smear with undissolved serum) differed somewhat from one another. For example, the common globulin isolated by the water-alcohol method (Cohn) from serum No 67 contained 0.169% protein in its working solution, while the euglobulin isolated by the water method contained 0.25% protein, i.e., its activity was 1.5 times lower. A similar difference in activity was observed with the euglobulin isolated form serum No 69.

As regards the antibodies (globulins) labelled with fluorescein isothiocyanate (II isomer), -- the reverse relationships prevailed here. Thus, in the anticholera aggluitnating serum of series No 67, the euglobulin obtained by the water method contained 0.015% protein in working solution, while the Cohn-method globulin contained 0.031% protein, i.e., the activity of the latter was twice as low.

In the case of agglutinating anti-plague serum No 5025, the activity of both globulins was the same (0.022 and 0.02%), which was apparently due to the similar method of labelled antibody purification.

Also noteworthy is the fact that the antibodies labelled with isothiocyanate produced bright glowing of the homologous bacteria with a protein content in the working solution which was 3-10 times lower than in the conjugates labelled with fluorescein isocyanate. This fact leads us to lend preference to the new dye -- fluorescein isothiocyanate.

In conclusion, it is necessary to note the lability of the euglobulins as manifested in the partial loss of sero-

logical (coloring) activity after 2 months of storage at 4-5°. This phenomenon can apparently be eliminated through proper storage conditions and the addition of certain stabilizers to the conjugates. These conditions are presently under study.

Relative Advantages of Fluorescent Antibodies Prepared in Various Ways

Initial serum	Method of globulin isolation	Fluorochrome used in labbling	Method of purification to remove dye excess	Relative advantages of fluoresent antibodies	
				Protein content (%)	Protein content in working solution (%)
Agglutinating anti-cholera series No 67	Water alcohol	Fluorescein isocyanate	Alcohol	1,69	0,169
	Water	"	Water	1,77	0,25
"	Water alcohol	Fluorescein isothiocyanate II isomer	Alcohol	0,63	0,031
	Water	"	Water	0,5	0,015
Agglutinating anti-plague series No 69	Water alcohol	Fluorescein isocyanate	Alcohol	1,59	0,063
	Water	"	Water	1,9	0,1
Agglutinating anti-plague No 5025 (rabbit)	Water alcohol	Fluorescein isothiocyanate	"	0,7	0,022
	Water	"	"	0,63	0,02
"	Water alcohol	"	Cohn water-alcohol method	-	-
	Water	"	Water	0,8	0,025

Conclusions

1. A new method for the isolation of immune euglobulins (antibodies) from agglutinating anti-plague and anti-cholera serums has been proposed, a new method of purifying the labelled antibodies from the fluorochrome excess has been presented.

2. With the new method of isolating and purifying antibodies, the time required to prepare luminescent conjugates is shortened from 3-4 weeks to 3-4 days.

3. The suggested method of preparing fluorochrome-labelled antibodies is realizable in any laboratory, since it requires no special equipment (refrigeration centrifuges and rooms).

4. The advantages of a new luminescent stain -- fluorescein isothiocyanate, over fluorescein isocyanate are shown.

5. The immune euglobulins isolated by the new method were classifiable among the γ-globulins in their electrophoretic composition.

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